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TITLE: Unlocking Barriers to DNA Vaccine Immunogenicity: A Cross-Species Analysis of Cytosolic DNA Sensing in Skeletal Muscle Myocytes

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14. ABSTRACT

DNA vaccine technology holds great promise as a platform for developing vaccines against both emerging and established global pathogens. Despite this potential, significant challenges impede the capacity of DNA vaccines to prevent disease in humans. Foremost amongst these, is the gap between remarkable results obtained in pre-clinical mouse models and relatively modest immunogenicity observed in humans. The present work is testing the hypothesis that skeletal muscle myocytes sense cytosolic DNA and elaborate an inflammatory response to DNA vaccines and that species-specific differences in the cytosolic DNA sensing system bring about divergent inflammatory responses in human versus mouse skeletal muscle myocytes. We are pursuing the following specific aims: 1) characterize the inflammatory response elaborated by myocytes following the delivery of DNA to the cytosol, 2) define the components of the cytosolic DNA sensing system that are present in skeletal muscle myocytes and 3) ascertain which components of the myocyte cytosolic DNA sensing system are engaged upon delivery of DNA to the cytosol. We are utilizing molecular, biochemical and proteomic methods to analyze the consequences of DNA vaccine vector delivery into mouse and human myocyte-derived cell lines and primary cells. We anticipate that our efforts will produce important insights on cytosolic DNA sensors and provide a key to unlock DNA vaccine immunogenicity for humans.

15. SUBJECT TERMS
DNA vaccine, skeletal muscle myocytes, cytosolic DNA sensor

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Unlocking Barriers to DNA vaccine Immunogenicity: A Cross-Species Analysis of Cytosolic DNA Sensing in Skeletal Muscle Myocytes

1. INTRODUCTION:

We speculate that the skeletal muscle myocyte cytosolic DNA surveillance system can influence both the expression of and immune response to DNA vaccine antigens, thus impacting upon the immunogenicity of DNA vaccines. Moreover, we speculate that key differences in the function of DNA sensors/adaptors in mouse versus human myocytes contribute to the marked cross-species differences observed in DNA vaccine immunogenicity. We are therefore pursuing the hypothesis that skeletal muscle myocytes sense cytosolic DNA and elaborate the initial inflammatory response to DNA vaccines. We are also pursuing the secondary hypothesis that species-specific functional differences in the cytosolic DNA surveillance system bring about a divergent inflammatory response in human versus mouse skeletal muscle myocytes. Our ongoing project is focused on three specific aims including: 1) to define the components of the cytosolic DNA surveillance system that are present in human and mouse skeletal muscle myocytes, 2) to characterize the inflammatory response elaborated by human and mouse myocytes following delivery of DNA to the cytosol and 3) to ascertain which components of the human and mouse myocytes' cytosolic DNA surveillance systems are engaged and essential for the induction of inflammatory responses. This annual report describes our efforts to date in accomplishing these scientific objectives.

2. KEYWORDS:

DNA vaccine, cytosolic DNA sensor, skeletal muscle myocytes

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Please note that the SOW has been updated to reflect changes in the proposed start and completion dates or duration of certain activities. We are continuing to pursue all major objectives and specific objectives as delineated in our original SOW. We have however split subtasks under each major task to reflect initiation of work with mouse myocytes prior to work with human myocytes. For example, if a subtask pertaining to both mouse and human cells was referred to as "Subtask 1" in the original SOW, it may now be split into "Subtask 1A" for mouse cell related work and "Subtask 1B" for human cell related work. The major goals set out below therefore reflect the revised SOW document which is also provided separately. Reference to the original SOW target dates and proportion of work accomplished for each subtask are also specified below. The proportion of each subtask completed pertains to work completed by the date of the original annual report (October 8, 2016).

Specific Aim 1: Define the components of the cytosolic DNA surveillance system that are present in human and mouse skeletal muscle myocytes

Major Task 1: Quantitative transcript analyses

Subtask 1A:
i) optimize culture and differentiation methods for **mouse myocyte cell lines** and evaluate phenotype of myocytes at different stages of differentiation using microscopy
ii) establish expression levels of **mouse** myogenic differentiation markers by RT-PCR and immunoblot
iii) establish expression levels of selected **mouse** cytosolic DNA sensors by RT-PCR

- Planned activity duration in original SOW: 2015 Q3 to Q4
- Planned activity duration in revised SOW: 2015 Q4 – 2016 Q3
- Proportion of subtask completed: 100%
- Comment: This subtask has been completed using the mouse myocyte cell line model C2C12.

Subtask 1B:
i) optimize culture and differentiation methods for **mouse myocyte primary cells** and evaluate phenotype of myocytes at different stages of differentiation using microscopy
ii) establish expression levels of **mouse** myogenic differentiation markers by RT-PCR and immunoblot
iii) establish expression levels of selected **mouse** cytosolic DNA sensors by RT-PCR

- Planned activity duration in original SOW: 2015 Q3 to Q4
- Planned activity duration in revised SOW: 2016 Q4 – 2017 Q1
- Proportion of subtask completed: 0%
- Comment: Delay in onset of this activity reflects a decision to optimize assays and techniques using cell lines, prior to working with primary cells that are more difficult to obtain and maintain.

Subtask 1C:
i) optimize culture and differentiation methods for **human myocytes cell lines and primary cells** & evaluate phenotype of myocytes at different stages of differentiation using microscopy
ii) establish expression levels of **human** myogenic differentiation markers by RT-PCR and immunoblot
iii) establish expression levels of selected **human** cytosolic DNA sensors by RT-PCR

- Planned activity duration in original SOW: 2015 Q3 to Q4
- Planned activity duration in revised SOW: 2016 Q4 – 2017 Q1
- Proportion of subtask completed: 0%

- Comment: Work with human cell lines was delayed due to technical difficulty expanding the cell line and cost of reagents. Accordingly, we are initiating work with human cell lines in Q4 of 2016 and human primary cells in Q1 of 2017.

Subtask 2A:
i) configure a NanoString assay panel with candidate **mouse** cytosolic DNA sensors/adaptors and other relevant genes
ii) evaluate NanoString assay panel performance using **mouse** monocyte/macrophage cell lines

- Planned activity duration in original SOW: 2016 Q1 to Q2
- Planned activity duration in revised SOW: 2016 Q4 – 2017 Q1
- Proportion of subtask completed: 0%
- Comment: This subtask has not been initiated. Given the relatively high cost of this technology, we will only proceed with its use in Q4 of 2016.

Subtask 2B:
i) configure a NanoString assay panel with candidate **human** cytosolic DNA sensors/adaptors and other relevant genes
ii) evaluate NanoString assay panel performance using **human** monocyte/macrophage cell lines

- Planned activity duration in original SOW: 2016 Q1 to Q2
- Planned activity duration in revised SOW: 2016 Q4 – 2017 Q1
- Proportion of subtask completed: 0%
- Comment: This subtask has not been initiated. Given the relatively high cost of this technology, we will only proceed with its use in Q4 of 2016.

Subtask 3A:
i) evaluate mouse myocytes using configured NanoString panels
ii) validate gene expression for a small number of selected transcripts of interest queried by real-time quantitative RT-PCR

- Planned activity duration in original SOW: 2016 Q1 to Q2
- Planned activity duration in revised SOW: 2016 Q4 – 2017 Q1
- Proportion of subtask completed: 0%
- Comment: This subtask has not been initiated. Given the relatively high cost of this technology, we will only proceed with its use in Q4 of 2016.

Subtask 3B:
i) evaluate human myocytes using configured NanoString panels
ii) validate gene expression for a small number of selected transcripts of interest queried by real-time quantitative RT-PCR

- Planned activity duration in original SOW: 2016 Q1 to Q2

- Planned activity duration in revised SOW: 2016 Q4 – 2017 Q1
- Proportion of subtask completed: 0%
- Comment: This subtask has not been initiated. Given the relatively high cost of this technology, we will only proceed with its use in Q4 of 2016.

Milestone: We will determine which cytosolic sensors/adaptors and downstream effectors are expressed in human and mouse skeletal muscle myocytes.

- Milestone not achieved at this time

Major Task 2: Protein detection by standard immunoblotting

Subtask 1A:
i) analyze **mouse** cell lysates for selected cytosolic DNA sensors/adaptors using immunoblotting techniques (with and without immunoprecipitation methods to enrich proteins)

- Planned activity duration in original SOW: 2016 Q1 to Q3
- Planned activity duration in revised SOW: 2016 Q1 – 2016 Q4
- Proportion of subtask completed: 70%

Subtask 1B:
i) analyze **human** cell lysates for selected cytosolic DNA sensors/adaptors using immunoblotting techniques (with and without immunoprecipitation methods to enrich proteins)

- Planned activity duration in original SOW: 2016 Q1 to Q3
- Planned activity duration in revised SOW: 2016 Q4 – 2017 Q1
- Proportion of subtask completed: 0%

Subtask 2A:
i) gauge expression and localization of selected **mouse** proteins by in-cell ELISA or quantitative immunofluorescence microscopy

- Planned activity duration in original SOW: 2016 Q3 to Q4
- Planned activity duration in revised SOW: 2016 Q2 – 2016 Q4
- Proportion of subtask completed: 30%

Subtask 2B:
i) gauge expression and localization of selected **human** proteins by in-cell ELISA or quantitative immunofluorescence microscopy

- Planned activity duration in original SOW: 2016 Q3 to Q4
- Planned activity duration in revised SOW: 2016 Q4 – 2017 Q1
- Proportion of subtask completed: 0%

Milestone: We will determine which cytosolic sensors/adaptors and downstream effectors are expressed in human and mouse skeletal muscle myocytes

- Milestone not achieved at this time

Specific Aim 2: Characterize the inflammatory response elaborated by human and mouse myocytes following delivery of DNA to the cytosol

Major Task 1: Transcription factor activation analysis

Subtask 1:
i) design and produce DNA vaccine vectors that incorporate a reporter protein and a viral antigen protein then verify by sequencing
ii) characterize expression using bioluminescence, immunofluorescence and immunoblotting methods

- Planned activity duration in original SOW: 2015 Q3 to Q4
- Planned activity duration in revised SOW: 2015 Q4 – 2016 Q3
- Proportion of subtask completed: 100%

Subtask 2A:
i) use multiplex probe-capture technique and analysis on a Luminex platform to ascertain which **mouse** transcription factors are activated subsequent to entry of DNA

- Planned activity duration in original SOW: 2016 Q1 to Q2
- Planned activity duration in revised SOW: 2016 Q4 – 2017 Q1
- Proportion of subtask completed: 0%

Subtask 2B:
i) use multiplex probe-capture technique and analysis on a Luminex platform to ascertain which **human** transcription factors are activated subsequent to entry of DNA

- Planned activity duration in original SOW: 2016 Q1 to Q2
- Planned activity duration in revised SOW: 2016 Q4 – 2017 Q1
- Proportion of subtask completed: 0%

Subtask 3A:
i) validate **mouse** cell findings using chemiluminescent electrophoretic mobility shift assays (or DNA-binding ELISA technique in 96-well format)

- Planned activity duration in original SOW: 2016 Q2 to Q3
- Planned activity duration in revised SOW: 2016 Q4 – 2017 Q1
- Proportion of subtask completed: 0%

Subtask 3B:
i) validate **human** cell findings using chemiluminescent electrophoretic mobility shift assays (or DNA-binding ELISA technique in 96-well format)

- Planned activity duration in original SOW: 2016 Q2 to Q3
- Planned activity duration in revised SOW: 2016 Q4 – 2017 Q1
- Proportion of subtask completed: 0%

Subtask 4A:
i) evaluate key **mouse** cell transcription factors NFκB, IRF-3, and IRF-7 separately by immunoblotting of cellular extracts with phospho-specific antibodies

- Planned activity duration in original SOW: 2016 Q2 to Q3
- Planned activity duration in revised SOW: 2016 Q4 – 2017 Q1
- Proportion of subtask completed: 0%

Subtask 4B:
i) evaluate key **human** cell transcription factors NFκB, IRF-3, and IRF-7 separately by immunoblotting of cellular extracts with phospho-specific antibodies

- Planned activity duration in original SOW: 2016 Q2 to Q3
- Planned activity duration in revised SOW: 2016 Q4 – 2017 Q1
- Proportion of subtask completed: 0%

Milestone: We will ascertain which transcription factors are activated subsequent to entry of DNA into the cytosol, for both human and mouse skeletal muscle myocytes

- Milestone not achieved at this time

Major Task 2: Secretome analysis by multiplex cytokine capture

Subtask 1
As for Major Task 1

Subtask 2A:
i) detect and quantify **mouse** cytokines in supernatants using panel of cytokine-specific antibodies coupled to spectral signature-unique fluorescent beads with Luminex

- Planned activity duration in original SOW: 2016 Q1 to Q2
- Planned activity duration in revised SOW: 2016 Q4 – 2017 Q1
- Proportion of subtask completed: 0%

Subtask 2B:
i) detect and quantify **human** cytokines in supernatants using panel of cytokine-specific antibodies coupled to spectral signature-unique fluorescent beads with Luminex

- Planned activity duration in original SOW: 2016 Q1 to Q2
- Planned activity duration in revised SOW: 2017 Q1
- Proportion of subtask completed: 0%

Subtask 3A:
i) validate specific mouse cell findings using immunoprecipitation with immunoblotting/ELISA
ii) further validate findings by utilizing immunofluorescence microscopy-based intracellular cytokine detection

- Planned activity duration in original SOW: 2016 Q2 to Q4
- Planned activity duration in revised SOW: 2016 Q4 – 2017 Q1
- Proportion of subtask completed: 0%

Subtask 3B:
i) validate specific human cell findings using immunoprecipitation with immunoblotting/ELISA
ii) further validate findings by utilizing immunofluorescence microscopy-based intracellular cytokine detection

- Planned activity duration in original SOW: 2016 Q2 to Q4
- Planned activity duration in revised SOW: 2017 Q1
- Proportion of subtask completed: 0%

Milestone: We will determine which cytokines are secreted, by both human and mouse skeletal muscle myocytes, subsequent to entry of DNA into the cytosol

- Milestone not achieved

Specific Aim 3: Ascertain which components of the human and mouse myocytes' cytosolic DNA surveillance systems are engaged and essential for the induction of inflammatory responses

Major Task 1: "In vivo" protein-protein & DNA-protein cross-linking pull-down

Subtask 1A:
i) optimize membrane permeable chemical cross-linker method with **mouse** cells
ii) perform co-immunoprecipitation (co-IP) assays to pull-down proteins complexes formed in DNA exposed vs. non-exposed myocytes
iii) perform "time lapse" experiments

- Planned activity duration in original SOW: 2015 Q4 to 2016 Q2
- Planned activity duration in revised SOW: 2016 Q4 – 2017 Q1
- Proportion of subtask completed: 0%

Subtask 1B:
i) optimize membrane permeable chemical cross-linker method with **human** cells
ii) perform co-immunoprecipitation (co-IP) assays to pull-down proteins complexes formed in DNA exposed vs. non-exposed myocytes
iii) perform "time lapse" experiments

- Planned activity duration in original SOW: 2015 Q4 to 2016 Q2
- Planned activity duration in revised SOW: 2017 Q1
- Proportion of subtask completed: 0%

Subtask 2A:
i) label plasmid DNA with photoactivatable biotin (e.g. Psoralen-PEG3-Biotin)
ii) perform DNA-protein complex pull-down method to pull-down DNA-protein complexes formed in DNA exposed vs. non-exposed **mouse** myocytes

- Planned activity duration in original SOW: 2015 Q4 to 2016 Q2
- Planned activity duration in revised SOW: 2016 Q4 – 2017 Q1
- Proportion of subtask completed: 0%

Subtask 2B:
i) label plasmid DNA with photoactivatable biotin (e.g. Psoralen-PEG3-Biotin)
ii) perform DNA-protein complex pull-down method to pull-down DNA-protein complexes formed in DNA exposed vs. non-exposed **human** myocytes

- Planned activity duration in original SOW: 2015 Q4 to 2016 Q2
- Planned activity duration in revised SOW: 2017 Q1
- Proportion of subtask completed: 0%

Subtask 3A:
i) use "shotgun" proteomics methods (solubilized and denatured proteins are separated, subjected to tandem mass spectrometry (MS) and analyzed for identification) to determine the constituents of protein-protein and DNA-protein complexes derived from **mouse** cells

- Planned activity duration in original SOW: 2016 Q1 to 2016 Q3
- Planned activity duration in revised SOW: 2017 Q1
- Proportion of subtask completed: 0%

Subtask 3B:
i) use "shotgun" proteomics methods (solubilized and denatured proteins are separated, subjected to tandem mass spectrometry (MS) and analyzed for identification) to determine the constituents of protein-protein and DNA-protein complexes derived from human cells

- Planned activity duration in original SOW: 2016 Q1 to 2016 Q3
- Planned activity duration in revised SOW: 2017 Q1
- Proportion of subtask completed: 0%

Subtask 4A:
i) confirm specific mouse cell findings using tandem affinity tag purification method whereby candidate partners are co-expressed as 3X FLAG and Twin-StrepTag fusion proteins in mouse cells and subsequently subjected to bead-based affinity purification and immunoblotting or ELISA based detection

- Planned activity duration in original SOW: 2016 Q3 to 2016 Q4
- Planned activity duration in revised SOW: 2017 Q1
- Proportion of subtask completed: 0%

Subtask 4B:
i) confirm specific human cell findings using tandem affinity tag purification method whereby candidate partners are co-expressed as 3X FLAG and Twin-StrepTag fusion proteins in human cells and subsequently subjected to bead-based affinity purification and immunoblotting or ELISA based detection

- Planned activity duration in original SOW: 2016 Q3 to 2016 Q4
- Planned activity duration in revised SOW: 2017 Q1
- Proportion of subtask completed: 0%

Milestone Achieved: We will discover which cytosolic DNA sensors/adaptors and downstream effectors are operational in human and mouse skeletal muscle myocytes

- Milestone not achieved at this time

Major Task 2: Genome Engineering of Myocyte Cell Lines by CRISPR

Subtask 1A:
i) design and produce mammalian expression plasmids encoding the Cas9 protein and specially designed gRNAs (that directs the nuclease to complementary mouse genomic target sequences)
ii) confirm Cas9 and green fluorescent protein (GFP) reporter production by plasmids

- Planned activity duration in original SOW: 2016 Q1 to 2016 Q2
- Planned activity duration in revised SOW: 2016 Q4 – 2017 Q1
- Proportion of subtask completed: 0%

Subtask 1B:
i) design and produce mammalian expression plasmids encoding the Cas9 protein and specially designed gRNAs (that directs the nuclease to complementary human genomic target sequences)
ii) confirm Cas9 and green fluorescent protein (GFP) reporter production by plasmids

- Planned activity duration in original SOW: 2016 Q1 to 2016 Q2
- Planned activity duration in revised SOW: 2016 Q4 – 2017 Q1
- Proportion of subtask completed: 0%

Subtask 2A:
i) produce mouse myocyte cell lines that have undergone gene disruption via a technique named CRISPR
ii) confirm gene disruption and GFP expression
iii) select multiple individual clones characterized with quantitative gene expression

- Planned activity duration in original SOW: 2016 Q2 to 2016 Q3
- Planned activity duration in revised SOW: 2016 Q4 – 2017 Q1
- Proportion of subtask completed: 0%

Subtask 2B:
i) produce human myocyte cell lines that have undergone gene disruption via a technique named CRISPR
ii) confirm gene disruption and GFP expression
iii) select multiple individual clones characterized with quantitative gene expression

- Planned activity duration in original SOW: 2016 Q2 to 2016 Q3
- Planned activity duration in revised SOW: 2016 Q4 – 2017 Q1
- Proportion of subtask completed: 0%

Subtask 3A:
i) evaluate mouse cell line phenotype upon exposure to cytosolic DNA, as assessed by secretome and transcription factor analyses as described in Specific Aim 2, using cell lines produced with confirmed loss of the genes of interest

- Planned activity duration in original SOW: 2016 Q3 to 2016 Q4
- Planned activity duration in revised SOW: 2017 Q1
- Proportion of subtask completed: 0%

Subtask 3B:
i) evaluate human cell line phenotype upon exposure to cytosolic DNA, as assessed by secretome and transcription factor analyses as described in Specific Aim 2, using cell lines produced with confirmed loss of the genes of interest

- Planned activity duration in original SOW: 2016 Q3 to 2016 Q4
- Planned activity duration in revised SOW: 2017 Q1
- Proportion of subtask completed: 0%

Milestone: We will discover which cytosolic DNA sensors/adaptors and downstream effectors are essential for responding to cytosolic DNA in human and mouse skeletal muscle myocytes

- Milestone not achieved at this time

What was accomplished under these goals?

This is an ongoing project. We are continuing to pursue all major objectives and specific objectives as delineated in our original SOW. In the work detailed below, we describe our success using the mouse myocyte cell line C2C12 as an initial model of myocyte growth and differentiation. Furthermore, we have optimized transfection techniques for this model and have readily demonstrated expression of a fluorescent reporter protein upon transfection. Work with the cytosolic DNA sensors Ifi204 and STING is also described below. We are particularly intrigued by our data suggesting that myocyte levels of STING are modulated soon after entry of DNA into the cytosol following transfection.

The following tasks were completed by the date of the original annual report (October 8, 2016):

1) We tested different culture conditions and media supplements, in order to optimize mouse skeletal muscle myoblast cell line (C2C12) culture and differentiation methods. Optimization was judged based on evaluation of myotube formation using phase contrast microscopy. We found that growth and differentiation media, DMEM + 10% FBS and DMEM +2% donor horse serum respectively, performed optimally without additional supplements such as insulin (**Figure 1**). Pyruvate in culture media did not have a detrimental effect on differentiation and therefore media containing pyruvate was used.

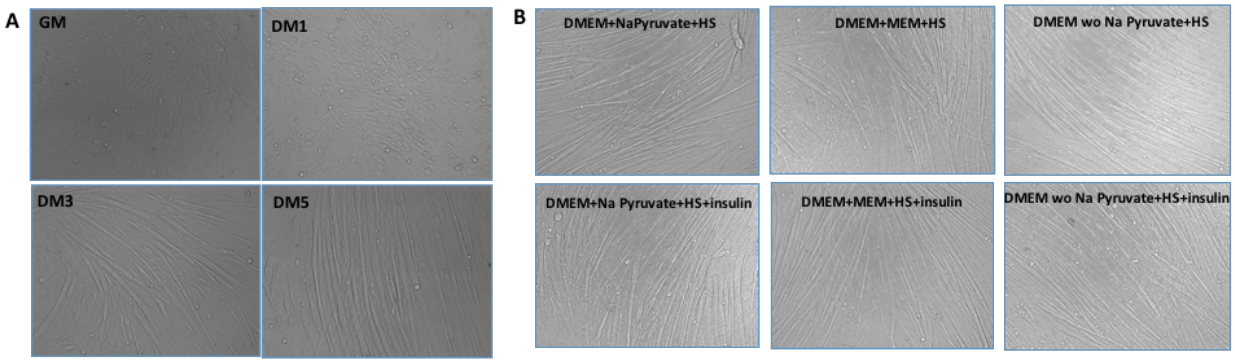

Figure 1. Evaluation of myotube formation using phase contrast microscopy. A. Optimal growth and differentiation with DMEM + 10% FBS (GM) and DMEM + 2% donor horse serum (DM), respectively. **B.** Test of different media combinations and conditions: DMEM +2% donor horse serum (HS) performed optimally for C2C12 differentiation without any additional supplements such as insulin or MEM. Pyruvate in culture media did not have a detrimental effect on differentiation.

2) Using immunoblotting methods, we established expression levels of myogenic differentiation markers (MyoD and MHC) in C2C12 cells at baseline, during differentiation and after transfection (**Figure 2**). Analyses using anti-MyoD monoclonal antibody (5.8A) demonstrated that MyoD is expressed in C2C12 cells before and after differentiation. Analyses using anti-MHC *fast* monoclonal antibody (MY-32) demonstrated that MHC expression levels increase with differentiation confirming that the differentiation process works. Furthermore, we observed that MyoD and MHC expression increases with differentiation after C2C12 transfection with pVAX-AcGFP.

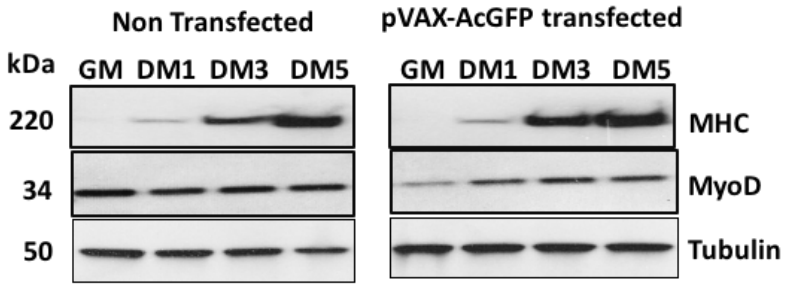

Figure 2. MyoD and MHC expression during differentiation and after transfection: Using anti-MyoD monoclonal antibody (5.8A) , we demonstrate that MyoD is expressed in C2C12 cells before and after differentiation. Analyses using anti-MHC *fast* monoclonal antibody (MY-32) demonstrated that MHC expression levels increase with differentiation.

3) We constructed a number of DNA expression vectors (plasmid based) in order to perform transfection and DNA vaccination experiments. These vectors include pVAX-mCherry (red fluorescent reporter), pVAX-AcGFP (green fluorescent reporter) and pVAX-p24 (HIV p24 protein). As these are all CMV promoter based mammalian expression vectors, we also utilized an alternate control AcGFP-expressing vector with an EF1alpha

promoter. A vector co-expressing HIV p24 protein as an antigen and AcGFP as reporter protein was also constructed. This was accomplished using only one promoter by introducing an intervening P2A "self-cleaving" peptide sequence between p24 and AcGFP. All vectors have been tested for expression in both HEK293T and C2C12 cells using fluorescence microscopy (**Figure 3A**) and immunoblotting methods (**Figures 3B and 3C**). In experiments aimed at detecting cytosolic DNA sensors, we have thus far utilized the pVAX-AcGFP vector.

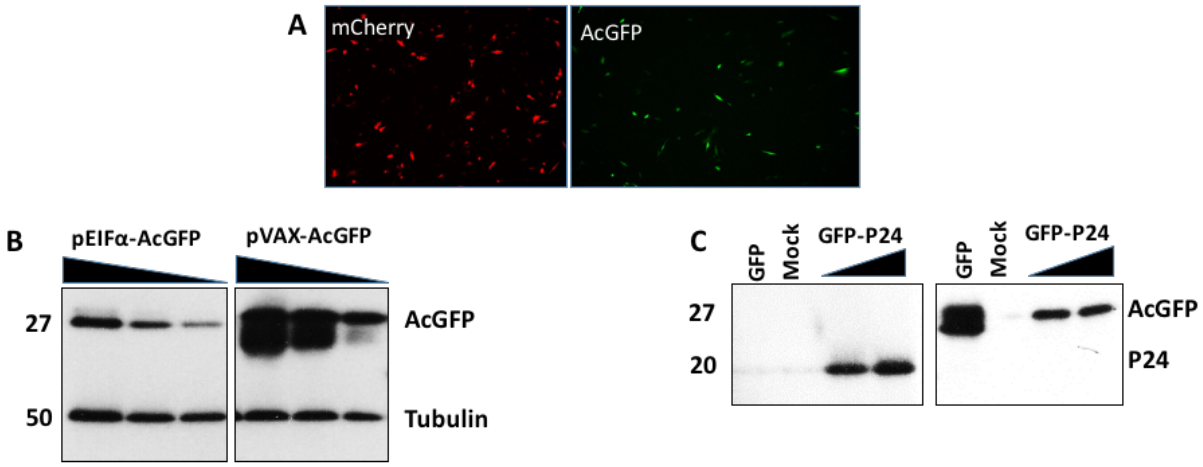

Figure 3. A. mCherry and GFP expression in Hek293 cells: We constructed a number of DNA expression vectors (plasmid based) in order to perform transfection and DNA vaccination experiments. These vectors include pVAX-mCherry (red fluorescent reporter) and pVAX-AcGFP (green fluorescent reporter). Live cell fluorescence microscopy images are shown. **B. Immunoblots of AcGFP expression in Hek293 cells**. Comparison of decreasing amounts of AcGFP-expressing vectors with a either a CMV promoter (pVAX) or an EF1alpha promoter (pEIFα) is shown. **C. Immunoblots of HIV p24 protein and AcGFP expression in Hek293 cells**. A single promoter vector co-expressing HIV p24 protein as an antigen and AcGFP as reporter protein was used to transfect HEk293 cells. The left side of the panel is probed with an anti-p24 specific antibody and the right side of the panel is probed with a anti-GFP-specific antibody.

4) Subsequent to optimization of our techniques, we found that C2C12 myoblasts could be readily transfected. However, upon differentiation, C2C12 myotubes were very difficult to transfect. A number of different transfection reagents and conditions were used with the same result. We also noted that while C2C12 myoblasts were efficiently transfected with pVAX-AcGFP, the expression of AcGFP was markedly diminished upon differentiation from myoblasts to myotubes (**Figure 4A and 4B**). This implied that differentiated myotubes somehow down-regulate expression from the DNA vector or degrade its product. Work to elucidate the mechanism behind this observation is ongoing.

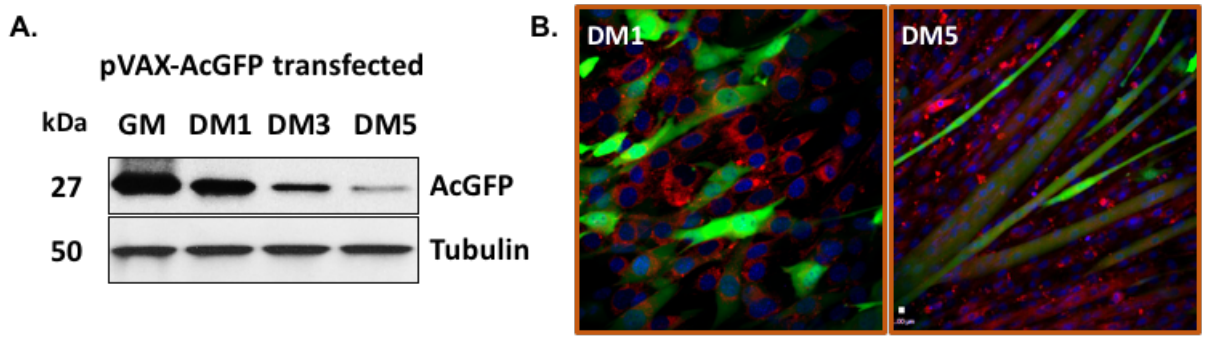

Figure 4. AcGFP expression after transfection and during C2C12 myoblasts differentiation. A. C2C12 myoblasts were efficiently transfected with pVAX-AcGFP as demonstrated by immunoblotting. Interestingly, the expression of AcGFP was markedly diminished upon differentiation from myoblasts to myotubes. **B**. Live cell fluorescence microscopy demonstrated AcGFP expression in pVAX-AcGFP transfected C2C12 cells at day one (DM1) and day five (DM5) post induction of differentiation.

5) Using immunoblotting methods, we observed that two cytosolic DNA sensors, STING and Ifi204, are expressed in C2C12 cells. The presence of these DNA sensors was therefore evaluated after transfection and during differentiation by both immunoblotting and RT-PCR methods. STING and Ifi204 proteins were expressed at the protein level in C2C12 cells at all stages of growth and differentiation (**Figure 5**).

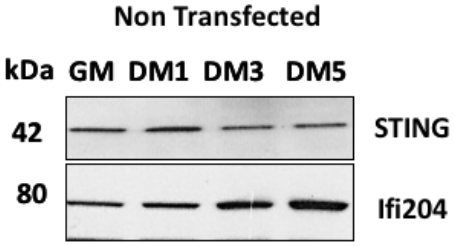

Figure 5. STING and Ifi204 expression during C2C12 myoblasts differentiation. STING expression did not seem to vary over the course of differentiation, however levels of ifi204 appeared to increase.

6) Using immunoblotting methods, we established expression levels of STING using anti-STING monoclonal antibody (D2P2F). STING protein was expressed in C2C12 cells in all stages of growth and differentiation. STING could no longer be detected by 48h after transfection of C2C12 cells with pVAX-AcGFP (**Figure 6A**). This implied that STING was either down-regulated or degraded shortly after interacting with cytosolic DNA or other sensors. Time course experiments subsequently revealed that the presence of STING is modulated as early as 2 hours after transfection with pVAX-AcGFP (**Figure 6B**), thus suggesting that the introduction of cytosolic DNA triggered degradation of STING.

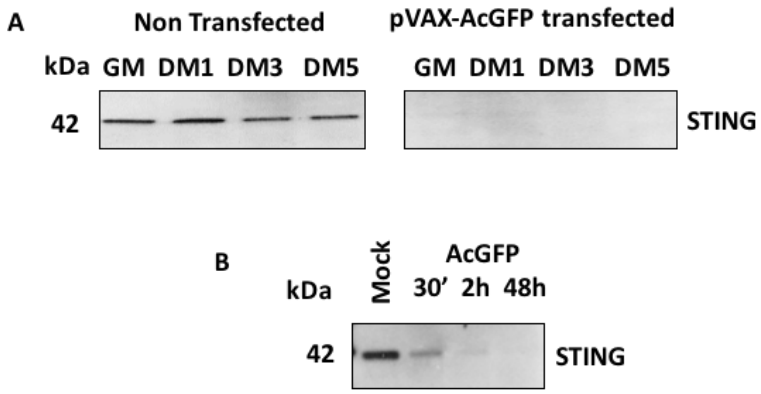

Figure 6. STING expression during differentiation and after transfection (GM represents 48h after transfection). **A.** STING could no longer be detected by 48h after transfection of C2C12 cells with pVAX-AcGFP. **B.** Time course experiments subsequently revealed that the presence of STING is modulated as early as 30 minutes after transfection with pVAX-AcGFP.

7) Using immunoblotting methods, we established expression levels of Ifi204 using anti-Ifi204 antibody. An anti Ifi204 polyclonal antibody (Thermofisher # PA5-23494) was used for this purpose. Analyses demonstrated that Ifi204 expression increases with C2C12 cell differentiation. We also observed that Ifi204 expression decreased after C2C12 transfection with pVAX-AcGFP (**Figure 7A**). Interestingly two bands were repeatedly seen migrating closely together on immunoblots on numerous independent experiments. A subsequent cellular fractionation method was optimized using nuclear and cytoplasmic protein markers and C1C12 cell nuclear and cytoplasmic extracts were analyzed by immunoblotting. In our initial series of experiments (using anti-Ifi204 polyclonal antibody, Lot# RC2171485) we observed one band of Ifi204 (>80kDa) exclusively present in the nuclear extract and one band (~80 kDa) exclusively present in the cytoplasmic extract. The signal intensity of the ~80kDa band present in the cytoplasmic extract increased with differentiation. Upon utilizing a new lot of antibody (Lot# RF2222416) only one band of Ifi204 (~80kDa) was visualized in both nuclear and cytosolic extracts (**Figure 7B**). The ~80kDa band signal intensity increased with differentiation in the cytoplasmic extract. Subsequently different lot numbers of anti-Ifi204 were tested and the results were comparable such that only one band at ~80 kDa was observed using both new and previously stored cell lysates. The manufacturer has been unable to explain this discrepancy.

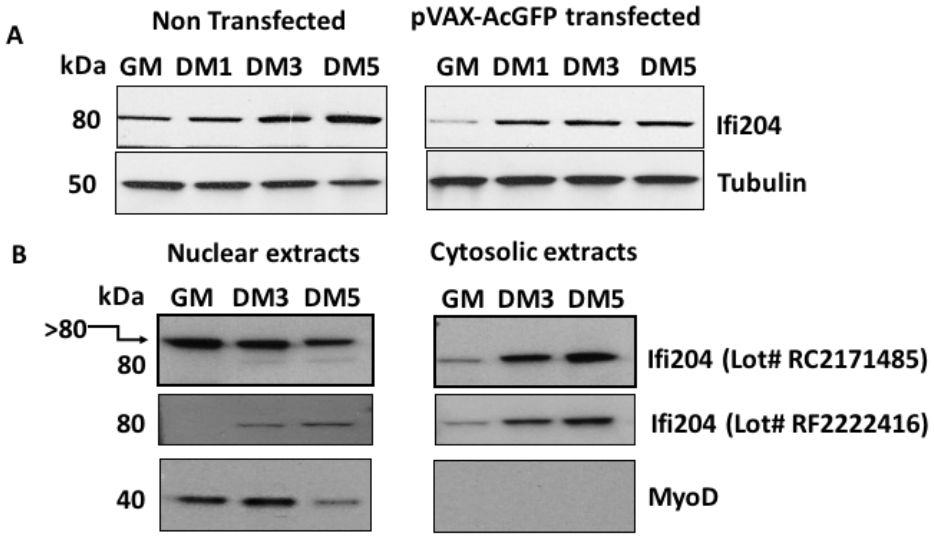

Figure 7. Ifi204 expression during differentiation and after transfection. A. Ifi204 expression increases with C2C12 cell differentiation. We also observed that Ifi204 expression increases in transfected cells after differentiation. **B.** Ifi204 expression in nuclear and cytosolic extracts with MyoD as nuclear control (bottom panels). Results with two different lots of anti-ifi204 specific antibody are shown. In the top panels, two isoforms are visualized. The larger isoform is exclusively present in nuclear extracts and the smaller isoform is predominantly present in cytoplasmic extracts.

8) Relative RNA expression level of STING and Ifi204 were also analyzed by RT-PCR. STING and Ifi204 RNA expression did not vary with transfection of C2C12 cells with pVAX-AcGFP, however, STING expression in pVAX-AcGFP transfected cells decreased with differentiation (**Figure 8**). A novel strategy was used to specifically detect Ifi204 transcripts, as standard RT-PCR methods initially resulted in significant non-specific amplification.

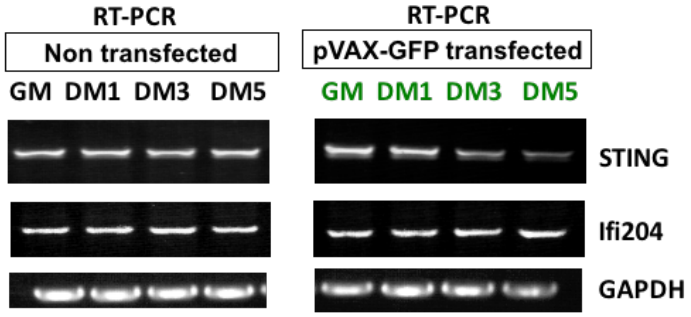

Figure 8. Relative RNA expression of Ifi204 and STING during differentiation and after transfection. In non-transfected C2C12 cells, STING and Ifi204 RNA expression did not vary over the course of differentiation. However, STING expression in pVAX-AcGFP transfected cells markedly decreased upon differentiation.

9) We have also initiated the following preliminary experiments: i) characterizing the inflammatory response in mouse myocytes following the delivery of DNA into the cytosol by transfection using an ELISA method to measure Interferon-beta secretion (**Figure 9**), ii) evaluating transcription factor (IRF-3, IRF-7 and NF kappa B) activation using immunoblotting methods and iii) ascertaining which components of the mouse myocytes' cytosolic DNA surveillance system are engaged in the induction of inflammatory responses using in vivo protein-protein cross-linking and immunoprecipitation methods in C2C12 (both non transfected and AcGFP transfected myoblasts).

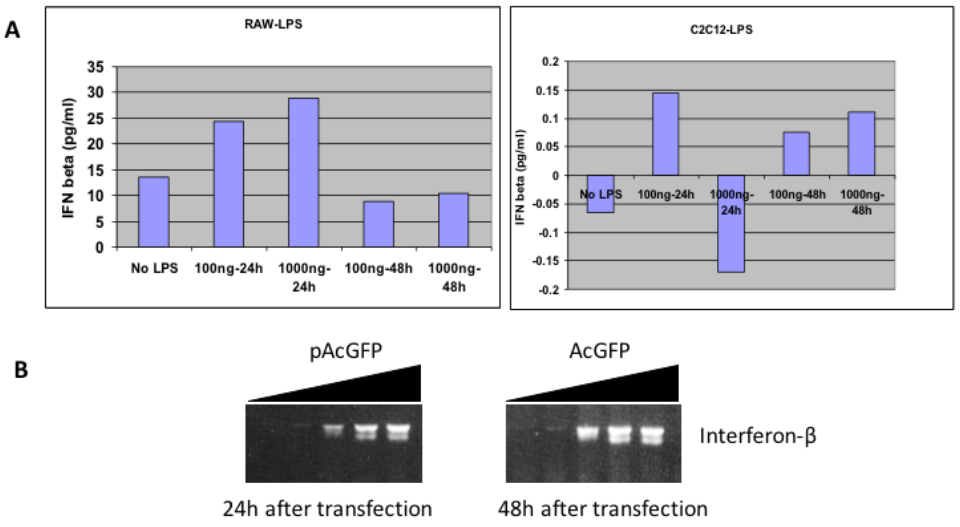

Figure 9. Induction of Interferon-beta expression in C2C12 cells with LPS or by transfection. A. Measurement of interferon-beta secretion by ELISA. RAW cells were used as control cells known to produce interferon-beta at basal level (left panel). LPS was only able to induce trace to negligible amounts of interferon-beta production in C2C12 (right panel). **B.** Interferon-beta expression by RT-PCR. Following the delivery of an increasing concentration of DNA into the cytosol by transfection, interferon-beta expression was induced with as little as 0.6ug of DNA.

What opportunities for training and professional development has the project provided?

The research personnel working on this project have received mentorship and additional training from the principal investigator with regard to general experimental design, DNA expression vector design, tissue culture, immunoblotting techniques, primer design and RT-PCR techniques. Training in fluorescent microscopy techniques has also been provided through the investigators core facility. Professional development activities in the form of weekly scientific seminars have also been provided to research personnel.

How were the results disseminated to communities of interest?

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

Laboratory personnel have now mastered all techniques required to accomplish the project and as such work towards attaining all objectives has accelerated. Work with human cells will begin shortly. We expect that the targeted approach we have taken thus far will guide subsequent work and allow us to use our resources more effectively in the last six months of the project. More advanced but costly techniques, likely to generate a large amount of data, are thus only now being used.

4. **IMPACT:**

What was the impact on the development of the principal discipline(s) of the project?

Nothing to Report

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. **CHANGES/PROBLEMS:**

Changes in approach and reasons for change

No changes in approach are anticipated. There are no changes to report with regard to the major goals of the project or scientific objectives. Please note however that work on this project began in Q4 of 2015, after funding was made available September 10th, 2015. The SOW listed Q3 of 2015 as the projected start for the initial phases of the project but this was delayed until contract negotiations and administrative tasks were completed. Personnel were hired at the end of September 2015.

Actual or anticipated problems or delays and actions or plans to resolve them

Problem 1: The research personnel originally listed in the grant submission in 2014 were no longer available in the investigators laboratory in Q4 of 2015. Accordingly, new personnel were hired. Postdoctoral Fellow Dr. Sonia Edaye started work on Sept 24th, 2015 and research technician Ms. Romina Gheorghe started work on Sept 28th, 2015. While highly qualified in some lab techniques, these individuals required additional training to attain competence in the techniques required to pursue the tasks set out in the SOW. Training was provided by the investigator and a former laboratory member and research personnel are now highly proficient. Work is now proceeding efficiently.

Problem 2: After discovering that the DNA sensor ifi204 (p204) was present in the C2C12 mouse myocyte cell line, we observed that two different protein isoforms were present and that the quantity of the larger protein isoform was modulated by the presence of cytosolic DNA. As this finding was exciting given its novelty and potential importance to our work, significant time and effort was expended pursuing further analyses including sub-cellular fractionation (nuclear and cytoplasmic). The larger protein isoform appeared to be enriched in the cell's nuclear fraction. We then sought to confirm this finding by establishing the presence of different transcript variants (i.e. differentially spliced transcripts). We only detected one transcript, thus arguing against the presence of different protein forms being produced by differentially spliced transcripts. Repeated experiments with a new lot of the antibody then failed to reveal two protein isoforms. Subsequent lots provided by the manufacturer have also failed to demonstrate two protein isoforms. Aliquots of the original lot were no longer available from the manufacturer. Accordingly, we assume that the antibody lot that was originally used was either contaminated or was produced in animals that received a different immunogen than present lots. We have been unable to get confirmation of either of these scenarios from the manufacturer. We also attempted to confirm our original findings with other ifi204 antibodies from different manufacturers without success. Work is now proceeding with new lots of antibody that have consistently shown only one protein isoform. We also optimized detection of mRNA transcripts using RT-PCR and we are now confident that only one isoform of ifi204 is present in myocytes. Once this issue was resolved, we proceeded with investigations of additional cytosolic DNA sensors (e.g. STING and cGAS).

Changes that had a significant impact on expenditures

Given the problems described above, greater than expected time, effort and expenditures were made during the first three quarters of the project. This work involved experiments analyzing cell lysates for cytosolic DNA sensors/adaptors using immunoblotting techniques. Accordingly, less was spent on using advanced but more costly techniques (e.g. Luminex and NanoString methods). We did however attempt to keep expenditures in check by initially working with cell lines rather than primary cells, as the latter system requires a greater use of resources. However, other than shifting some expenditures to later in the project timeline, overall we do not expect any impact on total expenditures required to complete the project.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals.

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. **PRODUCTS:**

Nothing to Report

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

What individuals have worked on the project?

Name:	**Dr. Gerasimos J. Zaharatos**
Project Role:	Principal Investigator
Researcher Identifier:	not registered
Nearest person month worked:	12
Contribution to Project:	• Provided training to personnel (postdoc and research technician) • Supervised all work • Designed overall research strategy • Guided experimental design • Assisted in analysis of data

Funding Support:	No other support

Name:	**Dr. Sonia Edaye**
Project Role:	Postdoctoral Fellow
Researcher Identifier:	not registered
Nearest person month worked:	12
Contribution to Project:	• Provided training to personnel (research technician) • Supervised work of research technician • Assisted in experimental design • Executed experiments • Analyzed data
Funding Support:	No other support

Name:	**Ms. Romina Gheorghe**
Project Role:	Research technician
Researcher Identifier:	not registered
Nearest person month worked:	12
Contribution to Project:	• Executed experiments • Assisted in analysis of data
Funding Support:	No other support

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Not applicable

QUAD CHARTS: Not applicable

9. **APPENDICES:**

The requested revision of the SOW is attached as an appendix on the following page and is comprised of six additional pages.

STATEMENT OF WORK – REVISED DECEMBER 3, 2016.
START DATE – SEPTEMBER 10, 2015.

JEWISH GENERAL HOSPITAL,
3755 COTE-STE-CATHERINE ROAD, ROOM E-0057
MONTREAL, QUEBEC, CANADA, H3T 1E2

PI: **GERASIMOS JERRY ZAHARATOS, MD**
COLLABORATOR/MENTOR: **MARK WAINBERG, PhD**

Title of Proposal: Unlocking Barriers to DNA Vaccine Immunogenicity: A Cross-Species Analysis of Cytosolic DNA Sensing in Skeletal Muscle Myocytes

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Research-Specific Tasks:

Specific Aim 1: Define the components of the cytosolic DNA surveillance system that are present in human and mouse skeletal muscle myocytes	**Year & Quarter (start to finish)**
Major Task 1: *Quantitative transcript analyses*	
Subtask 1A: i) optimize culture and differentiation methods for **mouse myocyte cell lines** and evaluate phenotype of myocytes at different stages of differentiation using microscopy ii) establish expression levels of **mouse** myogenic differentiation markers by RT-PCR and immunoblot iii) establish expression levels of selected **mouse** cytosolic DNA sensors by RT-PCR	**2015 Q4 – 2016 Q3**
Subtask 1B: i) optimize culture and differentiation methods for **mouse myocyte primary cells** and evaluate phenotype of myocytes at different stages of differentiation using microscopy ii) establish expression levels of **mouse** myogenic differentiation markers by RT-PCR and immunoblot iii) establish expression levels of selected **mouse** cytosolic DNA sensors by RT-PCR	**2016 Q4 – 2017 Q1**
Subtask 1C: i) optimize culture and differentiation methods for **human myocytes cell lines and primary cells** & evaluate phenotype of myocytes at different stages of differentiation using microscopy ii) establish expression levels of **human** myogenic differentiation markers by RT-PCR and immunoblot iii) establish expression levels of selected **human** cytosolic DNA sensors by RT-PCR	**2016 Q4 – 2017 Q1**

Subtask 2A: i) configure a NanoString assay panel with candidate **mouse** cytosolic DNA sensors/adaptors and other relevant genes ii) evaluate NanoString assay panel performance using **mouse** monocyte/macrophage cell lines	**2016 Q4 – 2017 Q1**
Subtask 2B: i) configure a NanoString assay panel with candidate **human** cytosolic DNA sensors/adaptors and other relevant genes ii) evaluate NanoString assay panel performance using **human** monocyte/macrophage cell lines	**2016 Q4 – 2017 Q1**
Subtask 3A: i) evaluate **mouse** myocytes using configured NanoString panels ii) validate gene expression for a small number of selected transcripts of interest queried by real-time quantitative RT-PCR	**2016 Q4 – 2017 Q1**
Subtask 3B: i) evaluate **human** myocytes using configured NanoString panels ii) validate gene expression for a small number of selected transcripts of interest queried by real-time quantitative RT-PCR	**2016 Q4 – 2017 Q1**
***Milestone Achieved:* We will determine which cytosolic sensors/adaptors and downstream effectors are expressed in human and mouse skeletal muscle myocytes**	
Major Task 2: *Protein detection by standard immunoblotting*	
Subtask 1A: i) analyze **mouse** cell lysates for selected cytosolic DNA sensors/adaptors using immunoblotting techniques (with and without immunoprecipitation methods to enrich proteins)	**2016 Q1 – 2016 Q4**
Subtask 1B: i) analyze **human** cell lysates for selected cytosolic DNA sensors/adaptors using immunoblotting techniques (with and without immunoprecipitation methods to enrich proteins)	**2016 Q4 – 2017 Q1**
Subtask 2A: i) gauge expression and localization of selected **mouse** proteins by in-cell ELISA or quantitative immunofluorescence microscopy	**2016 Q2 – 2016 Q4**
Subtask 2B: i) gauge expression and localization of selected **human** proteins by in-cell ELISA or quantitative immunofluorescence microscopy	**2016 Q4 – 2017 Q1**

Milestone Achieved: ***We will determine which cytosolic sensors/adaptors and downstream effectors are expressed in human and mouse skeletal muscle myocytes***	
Specific Aim 2: Characterize the inflammatory response elaborated by human and mouse myocytes following delivery of DNA to the cytosol	**Year & Quarter (start to finish)**
Major Task 1: *Transcription factor activation analysis*	
Subtask 1: i) design and produce DNA vaccine vectors that incorporate a reporter protein and a viral antigen protein then verify by sequencing ii) characterize expression using bioluminescence, immunofluorescence and immunoblotting methods	**2015 Q4 – 2016 Q3**
Subtask 2A: i) use multiplex probe-capture technique and analysis on a Luminex platform to ascertain which **mouse** transcription factors are activated subsequent to entry of DNA	**2016 Q4 – 2017 Q1**
Subtask 2B: i) use multiplex probe-capture technique and analysis on a Luminex platform to ascertain which **human** transcription factors are activated subsequent to entry of DNA	**2016 Q4 – 2017 Q1**
Subtask 3A: i) validate **mouse** cell findings using chemiluminescent electrophoretic mobility shift assays (or DNA-binding ELISA technique in 96-well format)	**2016 Q4 – 2017 Q1**
Subtask 3B: i) validate **human** cell findings using chemiluminescent electrophoretic mobility shift assays (or DNA-binding ELISA technique in 96-well format)	**2016 Q4 – 2017 Q1**
Subtask 4A: i) evaluate key **mouse** cell transcription factors NFκB, IRF-3, and IRF-7 separately by immunoblotting of cellular extracts with phospho-specific antibodies	**2016 Q4 – 2017 Q1**
Subtask 4B: i) evaluate key **human** cell transcription factors NFκB, IRF-3, and IRF-7 separately by immunoblotting of cellular extracts with phospho-specific antibodies	**2016 Q4 – 2017 Q1**
Milestone Achieved: ***We will ascertain which transcription factors are activated subsequent to entry of DNA into the cytosol, for both human and mouse skeletal muscle myocytes***	
Major Task 2: *Secretome analysis by multiplex cytokine capture*	
Subtask 1	**2015 Q4 – 2016 Q3**

As for Major Task 1	
Subtask 2A: i) detect and quantify **mouse** cytokines in supernatants using panel of cytokine-specific antibodies coupled to spectral signature-unique fluorescent beads with Luminex	**2016 Q4 – 2017 Q1**
Subtask 2B: i) detect and quantify **human** cytokines in supernatants using panel of cytokine-specific antibodies coupled to spectral signature-unique fluorescent beads with Luminex	**2017 Q1**
Subtask 3A: i) validate specific **mouse** cell findings using immunoprecipitation with immunoblotting/ELISA ii) further validate findings by utilizing immunofluorescence microscopy-based intracellular cytokine detection	**2016 Q4 – 2017 Q1**
Subtask 3B: i) validate specific **human** cell findings using immunoprecipitation with immunoblotting/ELISA ii) further validate findings by utilizing immunofluorescence microscopy-based intracellular cytokine detection	**2017 Q1**
Milestone Achieved: ***We will determine which cytokines are secreted, by both human and mouse skeletal muscle myocytes, subsequent to entry of DNA into the cytosol***	
Specific Aim 3: Ascertain which components of the human and mouse myocytes' cytosolic DNA surveillance systems are engaged and essential for the induction of inflammatory responses	**Year & Quarter (start to finish)**
Major Task 1: *"In vivo" protein-protein & DNA-protein cross-linking pull-down*	
Subtask 1A: i) optimize membrane permeable chemical cross-linker method with **mouse** cells ii) perform co-immunoprecipitation (co-IP) assays to pull-down proteins complexes formed in DNA exposed vs. non-exposed myocytes iii) perform "time lapse" experiments	**2016 Q4 – 2017 Q1**
Subtask 1B: i) optimize membrane permeable chemical cross-linker method with **human** cells ii) perform co-immunoprecipitation (co-IP) assays to pull-down proteins complexes formed in DNA exposed vs. non-exposed myocytes iii) perform "time lapse" experiments	**2017 Q1**

Subtask 2A: i) label plasmid DNA with photoactivatable biotin (e.g. Psoralen-PEG3-Biotin) ii) perform DNA-protein complex pull-down method to pull-down DNA-protein complexes formed in DNA exposed vs. non-exposed **mouse** myocytes	**2016 Q4 – 2017 Q1**
Subtask 2B: i) label plasmid DNA with photoactivatable biotin (e.g. Psoralen-PEG3-Biotin) ii) perform DNA-protein complex pull-down method to pull-down DNA-protein complexes formed in DNA exposed vs. non-exposed **human** myocytes	**2017 Q1**
Subtask 3A: i) use "shotgun" proteomics methods (solubilized and denatured proteins are separated, subjected to tandem mass spectrometry (MS) and analyzed for identification) to determine the constituents of protein-protein and DNA-protein complexes derived from **mouse** cells	**2017 Q1**
Subtask 3B: i) use "shotgun" proteomics methods (solubilized and denatured proteins are separated, subjected to tandem mass spectrometry (MS) and analyzed for identification) to determine the constituents of protein-protein and DNA-protein complexes derived from **human** cells	**2017 Q1**
Subtask 4A: i) confirm specific **mouse** cell findings using tandem affinity tag purification method whereby candidate partners are co-expressed as 3X FLAG and Twin-StrepTag fusion proteins in **mouse** cells and subsequently subjected to bead-based affinity purification and immunoblotting or ELISA based detection	**2017 Q1**
Subtask 4B: i) confirm specific **human** cell findings using tandem affinity tag purification method whereby candidate partners are co-expressed as 3X FLAG and Twin-StrepTag fusion proteins in **human** cells and subsequently subjected to bead-based affinity purification and immunoblotting or ELISA based detection	**2017 Q1**
***Milestone Achieved:* We will discover which cytosolic DNA sensors/adaptors and downstream effectors are operational in human and mouse skeletal muscle myocytes**	
Major Task 2: *Genome Engineering of Myocyte Cell Lines by CRISPR*	
Subtask 1A: i) design and produce mammalian expression plasmids encoding the Cas9 protein and specially designed gRNAs (that directs the nuclease to complementary **mouse** genomic target sequences)	**2016 Q4 – 2017 Q1**

ii) confirm Cas9 and green fluorescent protein (GFP) reporter production by plasmids	
Subtask 1B: i) design and produce mammalian expression plasmids encoding the Cas9 protein and specially designed gRNAs (that directs the nuclease to complementary **<u>human</u>** genomic target sequences) ii) confirm Cas9 and green fluorescent protein (GFP) reporter production by plasmids	**2016 Q4 – 2017 Q1**
Subtask 2A: i) produce **<u>mouse</u>** myocyte cell lines that have undergone gene disruption via a technique named CRISPR ii) confirm gene disruption and GFP expression iii) select multiple individual clones characterized with quantitative gene expression	**2016 Q4 – 2017 Q1**
Subtask 2B: i) produce **<u>human</u>** myocyte cell lines that have undergone gene disruption via a technique named CRISPR ii) confirm gene disruption and GFP expression iii) select multiple individual clones characterized with quantitative gene expression	**2016 Q4 – 2017 Q1**
Subtask 3A: i) evaluate **<u>mouse</u>** cell line phenotype upon exposure to cytosolic DNA, as assessed by secretome and transcription factor analyses as described in Specific Aim 2, using cell lines produced with confirmed loss of the genes of interest	**2017 Q1**
Subtask 3B: i) evaluate **<u>human</u>** cell line phenotype upon exposure to cytosolic DNA, as assessed by secretome and transcription factor analyses as described in Specific Aim 2, using cell lines produced with confirmed loss of the genes of interest	**2017 Q1**
<u>Milestone Achieved:</u> We will discover which cytosolic DNA sensors/adaptors and downstream effectors are essential for responding to cytosolic DNA in human and mouse skeletal muscle myocytes	